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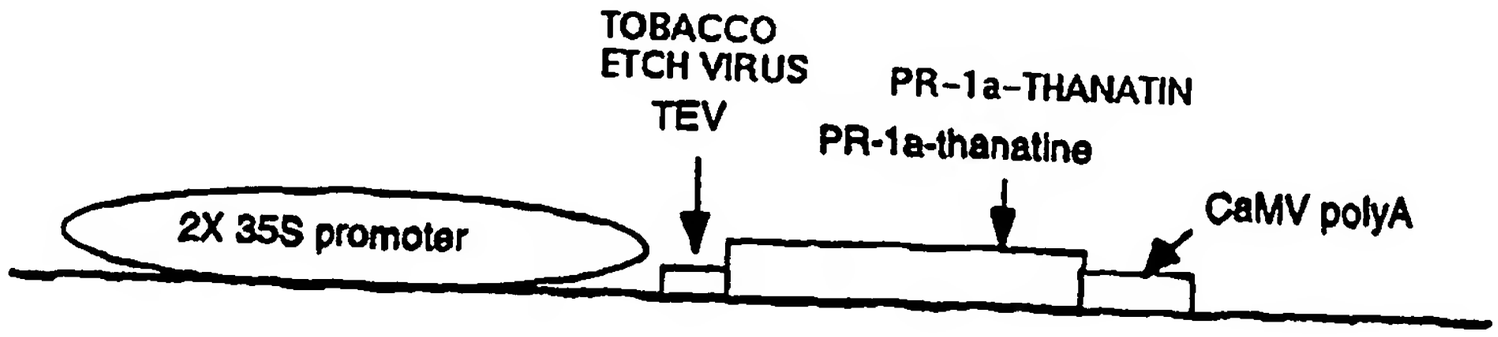
(54) Title: GENE CODING FOR THANATIN, VECTOR CONTAINING SAME AND RESULTING TRANSFORMED DISEASE-RESISTANT PLANTS

(54) Titre: GENE CODANT POUR LA THANATINE, VECTEUR LE CONTENANT ET PLANTES TRANSFORMEES OBTENUES RESISTANTES AUX MALADIES



(57) Abstract

The invention concerns a DNA sequence coding for thanatin, a vector containing it for transforming a host organism and the transformation method. More particularly the invention concerns the transformation of plant cells and plants, the drosomycin produced by the plants providing them with resistance to diseases, particularly of fungal origin.

(57) Abrégé

La présente invention a pour objet une séquence d'ADN codant pour la thanatine, un vecteur la contenant pour la transformation d'un organisme hôte et le procédé de transformation. L'invention concerne plus particulièrement la transformation des cellules végétales et des plantes, la drosomycine produite par les plantes transformées leur conférant une résistance aux maladies, en particulier d'origine fongique.

Gene encoding thanatin, vector containing it and disease-resistant transformed plants obtained

The subject of the present invention is a DNA sequence encoding thanatin, a vector containing it for the transformation of a host organism, and the method of transforming the said organism.

The invention relates more particularly to the transformation of plant cells and plants, the thanatin produced by the transformed plants conferring on them resistance to diseases, in particular of fungal origin.

An increasing need already exists for making plants resistant against diseases, in particular fungal diseases, in order to reduce, or even eliminate, the need for treatment with antifungal protection products, with a view to protecting the environment. One means of increasing this resistance to diseases consists in transforming the plants so that they produce substances capable of providing their defence against these diseases.

Various substances of natural origin, in particular peptides, are known which exhibit bactericidal or fungicidal properties, in particular against the fungi responsible for plant diseases. However, the problem consists in finding such

substances which not only can be produced by transformed plants, but can still preserve their bactericidal or fungicidal properties and confer them on the said plants. For the purposes of the present invention, bactericidal or fungicidal is understood to mean the actual bactericidal or fungicidal properties and the bacteriostatic and fungistatic properties.

Thanatin is a peptide produced by bacterial induction on adult *Psodius sp*, preferably *maculiventris*. Its preparation by bacterial induction is described in patent application FR 2,733,237, as well as its antifungal and antibacterial properties *in vitro*.

After having first identified the thanatin gene, it was also found that it could be inserted into a host organism, in particular a plant, in order to express the thanatin and confer on the said host organism properties of resistance to fungal diseases and to diseases of bacterial origin, providing a particularly advantageous solution to the problem stated above.

The subject of the invention is therefore first a nucleic acid fragment encoding thanatin, a chimeric gene comprising the said fragment encoding thanatin as well as heterologous regulatory elements at the 5′ and 3′ positions which can function in a host organism, in particular in plants, and a vector for transforming the host organisms containing this

chimeric gene, and the transformed host organism. It also relates to a transformed plant cell containing at least one nucleic acid fragment encoding thanatin and a disease-resistant plant containing the said cell, in particular regenerated from this cell. It finally relates to a method of transforming plants to make them resistant to diseases, in which method a gene encoding thanatin is inserted by means of an appropriate vector.

Thanatin is understood to mean according to the invention any peptide comprising essentially the peptide sequence of 11 amino acids which is described in patent application FR 2,733,237, as well as the equivalent homologous sequences in which certain amino acids are replaced by different but equivalent amino acids at sites which do not induce substantial modification of the antifungal or antibacterial activity of the said homologous sequence. Peptide sequence comprising essentially the peptide sequence described in patent application FR 2,733,237 is understood to mean not only the sequence defined by the sequence identifier No. 1 (SEQ ID NO 1), but also such a sequence comprising at either of its ends, or at both, peptide residues necessary for its expression and targeting in a host organism, in particular a plant cell or a plant.

Thanatin is a peptide of formula (I):

Xaa-Ile Ile Tyr Cys Asn Arg Arg Thr Gly Lys Cys-Xab

(I)

in which:

Xaa is NH_2 or a variable residue having a sequence comprising from 1 to 10 amino acids, and

Xab is OH or a variable residue having a sequence comprising from 0 to 5 amino acids.

Advantageously, when Xaa comprises at least one amino acid, the latter is one of the 20 base amino acids and more particularly chosen from the group comprising Gly, Ser, Lys, Pro and Val. When Xab comprises at least one amino acid, the latter is one of the 20 base amino acids and more particularly chosen from the group comprising Gln, Arg and Met.

According to a preferred embodiment of the invention, the two cysteine residues of the peptide of formula (I) form an intramolecular disulphide bridge.

The present invention therefore relates first to a nucleic acid, in particular a DNA, fragment encoding the thanatin defined above. It may be, according to the invention, a fragment isolated from *Psodius sp*, preferably *maculiventris*, or alternatively a derived fragment, suitable for the expression of thanatin in the host organism where the peptide will be expressed. The nucleic acid fragment may be obtained using standard methods of isolation and purification, or alternatively by synthesis according to the customary techniques of successive hybridizations of synthetic oligonucleotides. These techniques are in particular described by Ausubel et al.

According to the present invention, "nucleic acid fragment" is understood to mean a nucleotide sequence which may be of the DNA or RNA type, preferably of the DNA, in particular cDNA, especially double-stranded, type.

According to one embodiment of the invention, the nucleic acid fragment encoding thanatin comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO 1), a homologous sequence or a sequence complementary to the said sequence.

Advantageously, the nucleic acid fragment according to the invention comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO 2), a homologous sequence or a sequence complementary to the said sequence.

"Homologous" is understood to mean according to the invention a nucleic acid fragment having one or more sequence modifications relative to the nucleotide sequence described by the sequence identifier No. 1 or No. 2 and encoding thanatin. These modifications may be obtained according to the customary mutation techniques, or alternatively by choosing the synthetic oligonucleotides used in the preparation of the said sequence by hybridization. Given the multiple combinations of nucleic acids which may lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifier No. 1 or No. 2 and the homologue

may be great, especially since a DNA fragment of less than 100 nucleic acids in size, which can be produced by synthesis, is involved. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80%, more preferably at least 90%. These modifications are generally neutral, that is to say that they do not affect the primary sequence of the resulting thanatin.

The present invention also relates to a chimeric gene (or an expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5′ and 3′ positions which can function in a host organism, in particular plant cells or plants, the coding sequence comprising at least one DNA fragment encoding thanatin as defined above.

Host organism is understood to mean any higher or lower mono- or pluricellular organism into which the chimeric gene according to the invention may be introduced, for the production of thanatin. It consists of in particular bacteria, for example *E. coli*, yeasts, in particular of the genera *Saccharomyces* or *Kluyveromyces*, or preferably plant cells and plants.

"Plant cell" is understood to mean according to the invention any cell derived from a plant and which may constitute undifferentiated tissues such as calli, and differentiated tissues such as embryos, plant portions, plants or seeds.

"Plant" is understood to mean according to the invention any differentiated multicellular organism capable of photosynthesis, in particular monocotyledones or dicotyledones, more particularly cultivated plants intended or otherwise as animal feed or for human consumption, such as maize, wheat, colza, soya bean, rice, sugar cane, beet, tobacco, cotton and the like.

The regulatory elements necessary for the expression of the DNA fragment encoding thanatin are well known to persons skilled in the art depending on the host organism. They comprise in particular promoter sequences, transcription enhancers, transit peptides, terminator sequences, including start and stop codons. The means and methods for identifying and selecting the regulatory elements are well known to persons skilled in the art.

The nucleic acid fragment according to the invention may also comprise a nucleic acid sequence fused in 5′ and/or 3′ to the sequence encoding thanatin, so as to obtain a "protein-thanatin" fusion protein, whose cleavage by the enzymatic systems of the host organism allows the release of thanatin. This thanatin-fused protein may be a signal peptide or a transit peptide which makes it possible to control and orient the production of thanatin in a specific manner in a part of the host organism, such as for example the cytoplasm, the cell membrane, or in the case of plants

in a particular type of tissue or in the extracellular matrix.

According to one embodiment, the transit peptide may be a signal for chloroplast or mitochondrial addressing, which transit peptide is then cleaved in the chloroplast or the mitochondria.

According to another embodiment of the invention, the signal peptide may be an N-terminal signal or "prepeptide", optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a peptide for vacuolar addressing or "propeptide". The endoplasmic reticulum is the site where operations of maturation of the protein produced, such as for example the cleavage of the signal peptide, are carried out by the "cellular machinery".

The invention relates more particularly to the transformation of plants. As regulatory promoter sequence in plants, there may be used any promoter sequence of a gene which is expressed naturally in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for the small subunit of ribulose biscarboxylase (RuBisCO) or of a plant virus gene, for example that of cauliflower mosaic (CAMV 19S or 35S), or a promoter inducible by pathogens such as tobacco PR-1a or asparagus AoPRT-L, it being possible for any known suitable promoter to be used. Preferably, a regulatory

promoter sequence is used which promotes the overexpression of the coding sequence constitutively or inducibly by a pathogen attack, such as for example that comprising at least one histone promoter as described in application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence, other regulatory sequences which are situated between the promoter and the coding sequence, such as transcription enhancers such as for example the tobacco mosaic virus (TMV) translation enhancer described in application WO 87/07644, or the tobacco etch virus (TEV) translation enhancer described by Carrington & Freed, or transit peptides, either single or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in the direction of transcription, a sequence encoding a transit peptide of a plant gene encoding a plastid localization enzyme, a portion of sequence of the N-terminal mature portion of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide of a plant gene encoding a plastid localization enzyme consisting of a portion of sequence of the N-terminal mature portion of a plant gene encoding a plastid localization enzyme, as described in application EP 0,508,909. As transit peptide, there may be mentioned the signal peptide of the tobacco PR-1a gene described by Cornelissen et al.,

represented with its coding sequence by the sequence identifier No 3.

The sequence encoding the fusion protein signal peptide PR-1a-thanatin and this fusion protein also form part of the present invention. This sequence is in particular described by the sequence identifier No. 5, more particularly the coding part of this sequence, corresponding to bases 12 to 164.

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the nos terminator from *Agrobacterium tumefaciens*, or alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633,317.

According to the present invention, the chimeric gene may also comprise a selectable marker suitable for the transformed host organism. Such selectable markers are well known to persons skilled in the art. They may be a gene for resistance to antibiotics, such as penicillin, or alternatively a gene for tolerance of herbicides for plants.

The present invention also relates to a cloning or expression vector for the transformation of a host organism containing at least one chimeric gene as defined above. This vector comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid,

a bacteriophage or a virus, transformed by the introduction of the chimeric gene according to the invention. Such transformation vectors, depending on the host organism to be transformed, are well known to persons skilled in the art and are widely described in the literature.

For the transformation of plant cells or plants, they may consist in particular of a virus which may be used for the transformation of developed plants and containing, in addition, its own elements for replication and expression. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method of transforming host organisms, in particular plant cells, by integration of at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any known appropriate means widely described in the specialist literature and in particular the references cited in the present application, more particularly by the vector according to the invention.

A series of methods consists in bombarding cells or protoplasts with particles to which the DNA sequences are attached. Another series of methods consists in using, as means of transferring into the plant, a chimeric gene inserted into a Ti plasmid from

Agrobacterium tumefaciens or an Ri plasmid from *Agrobacterium rhizogenes*.

Other methods may be used, such as microinjection or electroporation or alternatively direct precipitation by means of PEG.

Persons skilled in the art will make the choice of the appropriate method depending on the nature of the host organism, in particular the plant cell or the plant.

The subject of the present invention is also the host organisms, in particular plant cells or plants, transformed and containing an effective quantity of a chimeric gene comprising a sequence encoding the thanatin defined above.

The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species, as for example described in the references above.

For the methods of transforming plant cells and of regenerating plants, the following patents and patent applications may be mentioned: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318,

US 5,204,253, US 5,405,765, EP 442,174, EP 486,233, EP 486,234, EP 539,563, EP 674,725, WO 91/02071 and WO 95/06128.

The present invention also relates to the transformed plants derived from the cultivation and/or crossing of the above regenerated plants, as well as the seeds of transformed plants.

The plants thus transformed are resistant to certain diseases, in particular to certain fungal or bacterial diseases. Consequently, the DNA sequence encoding thanatin may be integrated with the main objective of producing plants resistant to the said diseases, thanatin being effective against fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum* or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

The chimeric gene may also advantageously comprise at least one selectable marker, such as one or more herbicide tolerance genes.

The DNA sequence encoding thanatin may also be integrated as a selectable marker during the transformation of plants with other sequences encoding other peptides or proteins of interest such as, for example, herbicide tolerance genes.

Such herbicide tolerance genes are well known to a person skilled in the art and are in particular

described in patent applications EP 115 673, WO 87/04181, EP 337 899, WO 96/38567 or WO 97/04103.

Of course, the transformed cells and plants according to the invention may comprise, in addition to the sequence encoding thanatin, other heterologous sequences encoding other additional peptides capable of conferring on the plant resistance to other diseases of bacterial or fungal origin.

The other sequences may be integrated by means of the same vector comprising a chimeric gene, which comprises a first sequence encoding thanatin and at least one other sequence encoding another peptide or protein of interest.

They may also be integrated by means of another vector comprising at least the said other sequence, according to the customary techniques defined above.

The plants according to the invention may also be obtained by crossing parents, one carrying the gene according to the invention encoding thanatin, the other carrying a gene encoding at least one other peptide or protein of interest.

Among the sequences encoding other antifungal peptides, there may be mentioned that encoding drosomycin, which is described in patent application FR 2,725,992 and by Fehlbaum et al. (1994), and in the unpublished patent application FR 97 09115 filed on 24 July 1997, or that encoding androctonin described in

patent application FR 2,745,004 and in unpublished patent application FR 97 10362 filed on 20 August 1997.

The examples below make it possible to illustrate the invention, the preparation of the sequence encoding thanatin, of the chimeric gene, of the integration vector and of the transformed plants. Figures 1 to 5 in the annex describe the schematic structures of some plasmids prepared for the construction of chimeric genes. In these figures, the different restriction sites are marked in *italics*.

Example 1: **Construction of the chimeric genes**

All the techniques used below are standard laboratory techniques. The detailed protocols of these techniques are in particular described in Ausubel et al.

pRPa-MD-P: **Creation of a plasmid containing the signal peptide of the tobacco PR-1a gene**

The two complementary synthetic oligonucleotides Oligo 1 and Oligo 2 below are hybridized at 65°C for 5 minutes and then by slowly reducing the temperature to 30°C for 30'.

Oligo 1: 5' GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC ATCTTTCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'

Oligo 2: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'

After hybridization between Oligo 1 and Oligo 2, the DNA remaining single-stranded serves as a template for the Klenow fragment of polymerase I of *E. coli* (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes *SacII* and *NaeI* and cloned into the plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone is then obtained comprising the region encoding the signal peptide of the tobacco PR-1a gene (SEQ ID NO 3).

pRPA-PS-PR1a-than: Creation of a sequence encoding thanatin fused to the signal peptide PR-1a without a nontranscribed region in 3'

The two synthetic oligonucleotides with complementary sequences Oligo 3 and Oligo 4 based on the operating conditions described for **pRPA-MD-P**.

Oligo 3: 5' GGTTCCAAGA AGCCAGTGCC AATCATCTAC TGCAACAGGA CG 3'

Oligo 4: 5' CCGGATCCGT CGACACGTTC GCCTCGCCGA GCTCACATCC TCTGGCACTT ACCAGTCCTC CTGTTGCAGT AGATGATTGG CACTGGC 3'

After hybridization between Oligo 3 and Oligo 4, the DNA remaining single-stranded serves as a template for the Klenow fragment of polymerase I of *E. coli* (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. This double-stranded oligonucleotide containing the coding part of thanatin (SEQ ID NO 1) is then directly cloned into the plasmid pRPA-MD-P which has been digested with the restriction enzyme *NaeI*. The correct orientation of the clone obtained is checked by sequencing. A clone is then obtained comprising the region encoding the fusion protein PR-1a-thanatin situated between the *NcoI* restriction sites at the N-terminal end and the *ScaI, SacII* and *BamHI* restriction sites at the C-terminal end (SEQ ID NO 4).

<u>pRPA-RD-229</u>: Creation of an expression vector in plants comprising the sequence encoding the fusion protein PR-1a-thanatin

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr Jim Carrington (Texas A&M University, not described). This plamid, whose schematic structure is represented in Figure 1,

contains the double CaMV 35S promoter isolated from the cauliflower mosaic virus (CaMV 2x35S promoter, Odell et al., 1985) which directs the expression of an RNA containing [lacuna] tobacco etch virus 5' untranslated sequence (TEV 5' UTR; Carrington & Freed, 1990), the *E.coli* β-glucorinidase gene (GUS; Jefferson et al., 1987) followed by the polyadenylation site of the CaMV 35S RNA (CaMV polyA; Odell et al., 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI*, and the large DNA fragment is purified. The plasmid pRPA-PS-PR1a-than is digested with the restriction enzymes *NcoI* and *BamHI*, and the small DNA fragment containing the region encoding the fusion protein PR-1a-thanatin is purified. The two DNA fragments purified are then ligated together in an expression cassette in plants which synthesizes a PR-1a-thanatin fusion protein. The schematic structure of this expression cassette is represented in Figure 2. "PR-1a-thanatin" represents the coding region for the fusion protein PR-1a-thanatin of pRPA-RD-230. The thanatin is transported to the extracellular matrix of the plant by the action of the signal peptide PR-1a.

<u>pRPA-RD-195</u>: Creation of a plasmid containing a modified multiple cloning site

The plasmid pRPA-RD-195 is a plasmid derived from pUC-19 which contains a modified multiple cloning

site. The complementary synthetic oligonucleotides Oligo 5 and Oligo 6 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 5: 5′ AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG CATGC 3′

Oligo 6: 5′ CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC TAGAGG 3′

The double-stranded oligonucleotide obtained is then ligated into pUC-19 which has been previously digested with the restriction enzymes *EcoRI* and *HindIII* and made blunt ended using the Klenow fragment of DNA polymerase I of *E. coli*. A vector is obtained containing multiple cloning sites in order to facilitate the introduction of expression cassettes into an *Agrobacterium tumefaciens* vector plasmid. The schematic structure of this multiple cloning site is represented in Figure 3.

pRPA-RD-232: Introduction of the PR-1a-thanatin expression cassette of pRPA-RD-229 into pRPA-RD-195

The plasmid pRPA-RD-230 is digested with the restriction enzyme *HindIII*. The DNA fragment containing the PR-1a-thanatin expression cassette is purified. The

purified fragment is then ligated into pRPA-RP-195 which has previously been digested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase.

pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the bromoxynil tolerance gene of pRPA-BL-237 (EP 0,508,909)

The bromoxynil tolerance gene is isolated from pRPA-BL-237 by a PCR gene amplification. The fragment obtained is blunt-ended and is cloned into the *EcoRI* site of pRPA-BL-150A which has been made blunt-ended by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector is obtained which contains the bromoxynil tolerance gene close to its right border, a kanamycin tolerance gene close to its left border and a multiple cloning site between these two genes.

The schematic structure of pRPA-RD-174 is represented in Figure 4. In this figure, "nos" represents the *Agrobacterium tumefaciens* nopaline synthase polyadenylation site (Bevan et al., 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT II" represents the neomycin phosphotransferase gene of the *E. coli* Tn5 transposon (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from the cauliflower mosaic virus (Odell et al., 1985), "BRX"

represents the nitrilase gene isolated from *K. ozaenae* (Stalker et al., 1988), and "RB" and "LB" represent respectively the right and left borders of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

pRPA-RD-184: Addition of a new unique restriction site into pRPA-RD-174

The complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 7: 5′ CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
TACCTGGTTC AGG 3′

Oligo 8: 5′ CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
CACCTAGGCG CGCCGGGGCC GCGTTTAAAC TTAATTAAGT
GTGGCCTGAC TGG 3′

The double-stranded oligonucleotide hybridized (95 base pairs) is purified after separation on agarose gel (3% Nusieve, FMC). The plasmid pRPA-RD-174 is digested with the restriction enzyme *XmaI*, and the large DNA fragment is purified. The two DNA fragments obtained are then ligated.

A plasmid derived from pRPA-RD-174 is obtained comprising other restriction sites between the

bromoxynil tolerance gene and the selectable marker kanamycin gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 5 where the terms "nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB" and "LB" have the same meaning as in Figure 4.

pRPA-RD-235: Creation of an *Agrobacterium tumefaciens* vector containing the gene construct encoding thanatin directed towards the extracellular matrix

The plasmid pRPA-RD-232 is digested with the restriction enzymes *PmeI* and *AscI*, and the DNA fragment containing the gene for PR-1a-thanatin is purified. The plasmid pRPA-RD-184 is digested with the same restriction enzymes. The DNA fragment containing the PR-1a-thanatin expression cassette is then ligated into pRPA-RD-184. An *Agrobacterium tumefaciens* vector is thus obtained containing the sequence encoding the fusion protein PR-1a-thanatin which leads to the expression of thanatin in the plant's extracellular matrix.

Example 2: Herbicide tolerance of transformed tobaccos

2.1 - Transformation

The vector pRPA-RD-235 is introduced into the *Agrobacterium tumefaciens* EHA101 strain (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al.,

1986). The transformation technique is based on the procedure of Horsch et al. (1985).

2.2 - Regeneration

The regeneration of the tobacco PBD6 (origin SEITA France) from foliar explants is carried out on a Murashige and Skoog (MS) basal medium comprising 30 g/l of sucrose as well as 200 µg/ml of kanamycin. The foliar explants are removed from plants cultivated in a greenhouse or *in vitro* and regenerated according to the foliar disc technique (Horsh et al., 1985) in three successive stages: the first comprises the induction of shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed during this stage are then developed for 10 days by cultivating on an MS medium supplemented with 30 g/l of sucrose but containing no hormones. Next, the developed shoots are removed and they are cultivated on an MS rooting medium with half the content of salts, vitamins and sugar and containing no hormone. After about 15 days, the rooted shoots are transferred into the soil.

2.3 - Bromoxynil tolerance

Twenty transformed plants were regenerated and transferred into a greenhouse for the pRPA-RD-235 construct. These plants were then treated in a greenhouse at the 5-leaf stage with an aqueous

suspension of Pardner corresponding to 0.2 kg of bromoxynil active material per hectare.

All the plants showing complete tolerance to bromoxynil are then used in various experiments which show that the expression of thanatin by the transformed plants makes them resistant to fungal attacks.

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CLAIMS

1. Nucleic acid fragment, characterized in that it comprises a nucleic acid sequence encoding thanatin.

2. Nucleic acid fragment according to claim 1, characterized in that it is a DNA-type nucleotide sequence.

3. Nucleic acid fragment according to claim 2, characterized in that the DNA-type nucleotide sequence comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO 1), a homologous sequence or a sequence complementary to the said sequence.

4. Nucleic acid fragment according to claim 3, characterized in that the DNA-type nucleotide sequence comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO 2), a homologous sequence or a sequence complementary to the said sequence.

5. Nucleic acid fragment according to one of claims 1 to 4, characterized in that it comprises a nucleic acid sequence fused in 5′ and/or in 3′ to the sequence encoding thanatin, so as to obtain a "protein-thanatin" fusion protein.

6. Nucleic acid fragment according to claim 5, characterized in that the protein is a signal peptide or a transit peptide.

7. Nucleic acid fragment according to claim 6, characterized in that the signal peptide is the signal peptide of the tobacco PR-1a gene.

8. Nucleic acid fragment according to claim 7, characterized in that it comprises the DNA sequence described by the sequence identifier No. 5 (SEQ ID NO 5), a homologous sequence or a sequence complementary to the said sequence.

9. Nucleic acid fragment according to claim 8, characterized in that it comprises the coding part of SEQ ID NO 5, corresponding to bases 12 to 164.

10. Fusion protein "protein-thanatin", characterized in that the protein is a signal peptide or a transit peptide.

11. Fusion protein according to claim 10, characterized in that the signal peptide is the signal peptide of the tobacco PR-1a gene.

12. Fusion protein according to claim 11, characterized in that it is described by the sequence identifier No. 5 (SEQ ID NO 5).

13. Chimeric gene comprising a coding sequence as well as heterologous regulatory elements at the 5′ and 3′ positions which can function in a host organism, in particular plants, characterized in that

the coding sequence comprises at least one DNA fragment encoding thanatin as defined in claims 1 to 9.

14. Chimeric gene according to claim 13, characterized in that the host organism is chosen from plant cells and plants.

15. Chimeric gene according to either of claims 13 and 14, characterized in that it also comprises a selectable marker.

16. Cloning or expression vector for the transformation of a host organism, characterized in that it comprises at least one replication origin and at least one chimeric gene as defined in claims 13 to 15.

17. Vector according to claim 16, characterized in that it is a virus used for the transformation of developed plants and containing, in addition, its own elements for replication and expression.

18. Vector according to claim 16, characterized in that it is a plasmid.

19. Transformed host organisms, characterized in that they contain an effective quantity of a chimeric gene according to claims 13 to 15.

20. Transformed host organism according to claim 19, characterized in that it consists of plant cells or plants.

21. Transformed host organism according to claim 20, characterized in that it is a plant containing transformed cells.

22. Host organism according to claim 21, characterized in that the plant is regenerated from transformed cells.

23. Transformed plant cell, characterized in that it contains a nucleic acid fragment according to claims 1 to 9, or a chimeric gene according to claims 13 to 15.

24. Transformed plant resistant to diseases, characterized in that it comprises at least one transformed plant cell according to claim 23.

25. Transformed plant according to claim 24, characterized in that it is resistant to diseases caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum* or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

26. Disease-resistant transformed plant, characterized in that it is derived from the cultivation and/or crossing of plants according to either of claims 24 and 25.

27. Seeds of transformed plants according to one of claims 24 to 26.

28. Method of transforming host organisms, in particular plant cells or plants, characterized in

that at least one nucleic acid fragment according to claims 1 to 9 or a chimeric gene according to one of claims 13 to 15 is inserted into the said host organism.

29. Method of transforming plants to make them resistant to fungal or bacterial diseases, characterized in that at least one nucleic acid fragment according to claims 1 to 9 or a chimeric gene according to claims 13 to 15 is inserted into the plant.

30. Method according to either of claims 28 and 29, characterized in that the chimeric gene is inserted by means of a vector according to one of claims 16 to 18.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 13

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATC ATC TAC TGC AAC AGG AGG ACT GGT AAG TGC 33

Ile Ile Tyr Cys Asn Arg Arg Thr Gly Lys Cys

1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..63

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
GGT TCC AAG AAG CCA GTG CCA ATC ATC TAC TGC AAC AGG AGG ACT GGT        48
Gly Ser Lys Lys Pro Val Pro Ile Ile Tyr Cys Asn Arg Arg Thr Gly
 1               5                  10                  15
AAG TCG CAG AGG ATG
Lys Cys Gln Arg Met
            20
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..63

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GGT TCC AAG AAG CCA GTG CCA ATC ATC TAC TGC AAC AGG AGG ACT GGT        48
Gly Ser Lys Lys Pro Val Pro Ile Ile Tyr Cys Asn Arg Arg Thr Gly
 1               5                  10                  15
AAG TGC CAG AGG ATG TGAGCTCGGC GAGGCGAACG TGTCGACGGA TCCGG            98
Lys Cys Gln Arg Met
            20
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 106 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:12..101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGTCGACGC C ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT 50

Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu

1 5 10

CTT GTG TCT ACT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT 98

Leu Val Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg

15 20 25

GCC GGCGA 106

Ala

30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 197 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:12..164

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCGTCGACGC C ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT 50

Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu

1 5 10

CTT GTG TCT AT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT 98

Leu Val Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg

15 20 25

GCC GGT TCC AAG AAG CCA GTG CCA ATC ATC TAC TGC AAC AGG AGG ACT 146

Ala Gly Ser Lys Lys Pro Val Pro Ile Ile Tyr Cys Asn Arg Arg Thr

30 35 40 45

GGT AAG TGC CAG AGG ATG TGAGCTCGGC GAGGCGAACG TGTCGACGGA TCC 197

Gly Lys Cys Gln Arg Met

50

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 75 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC ATCTTTCCTT CTTGTGTCTA 60

CTCTTCTTCT TTTCC

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG 60

AAAGATGGAA GC 72

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTTCCAAGA AGCCAGTGCC AATCATCTAC TGCAACAGGA CG 42

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 97 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGGATCCGT CGACACGTTC GCCTCGCCGA GCTCACATCC TCTGGCACTT ACCAGTCCTC 60

CTGTTGCAGT AGATGATTGG CACTGGC 87

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 85 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC 60

CTCTAGAGTC GACCTGCAGG CATGC 85

(2) INFORMATION FOR SEQ ID NO: 110:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 6"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC TAGAGG 60

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 7"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC CCCGGCGCGC CTAGGTGTGT 60

GCTCGAGGGC CCAACCTCAG TACCTGGTTC AGG 93

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 8"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

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GCGTTTAAAC TTAATTAAGT GTGGCCTGAC TGG 93

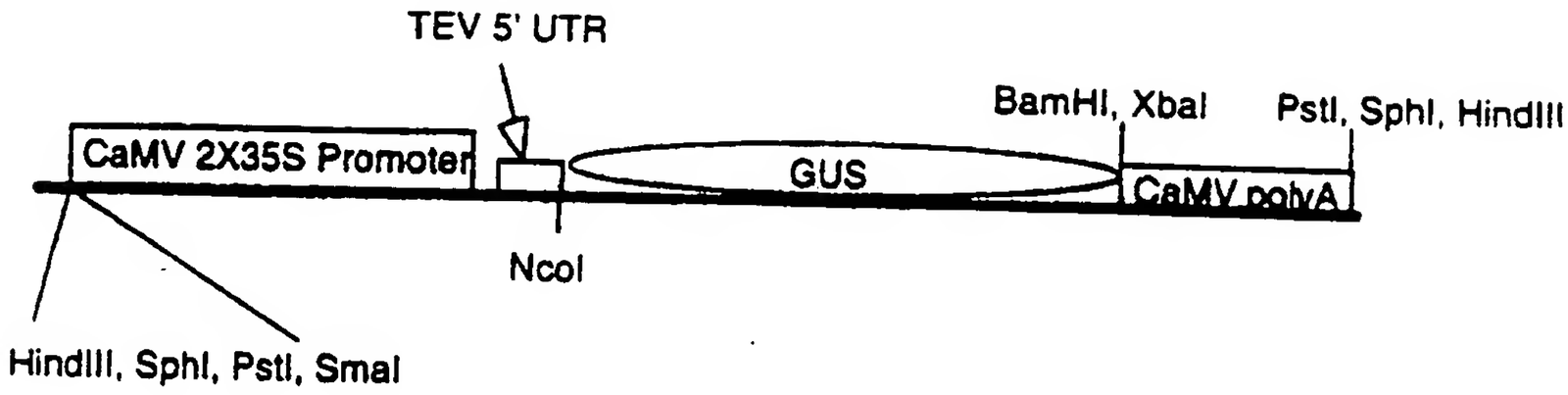

Fig. 1

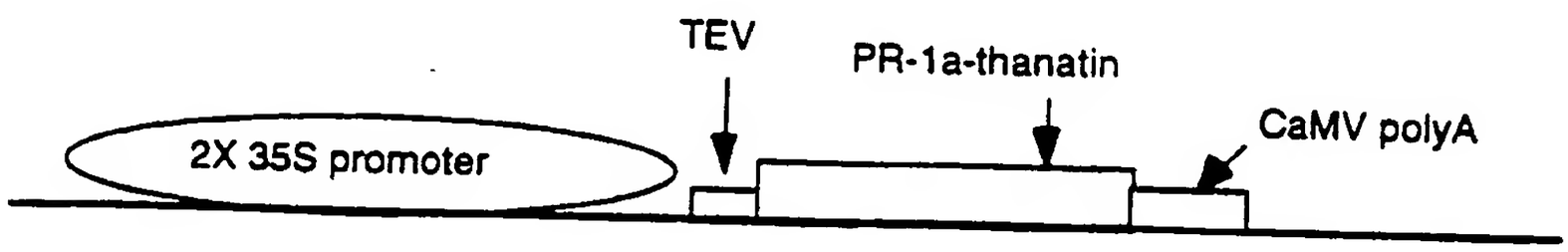

Fig. 2

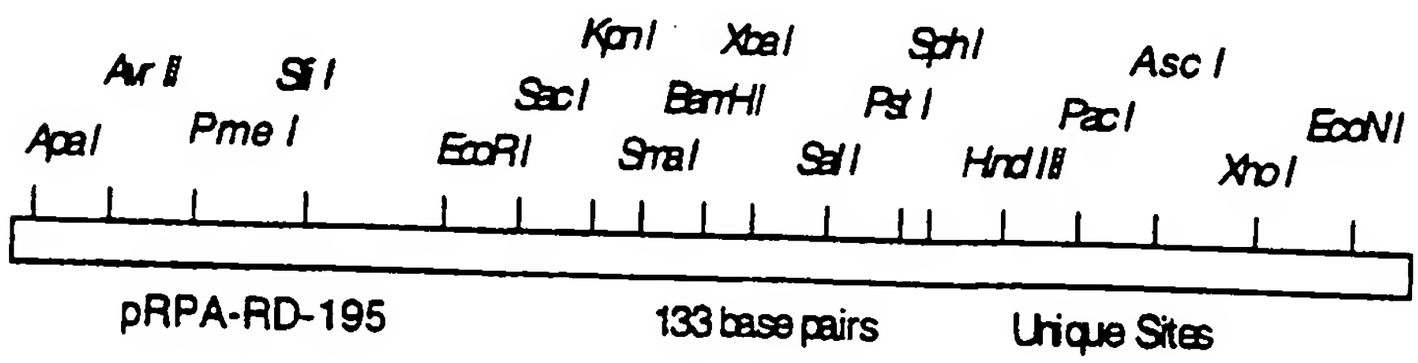

Fig. 3

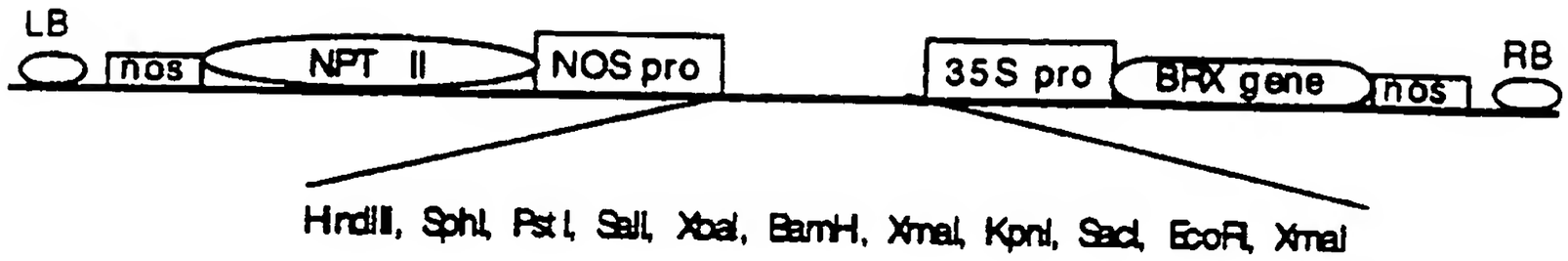
LB
nos
NPT II
NOS pro
35S pro
BRX gene
nos
RB
HindIII, SphI, PstI, SalI, XbaI, BamHI, XmaI, KpnI, SacI, EcoRI, XmaI

Fig. 4

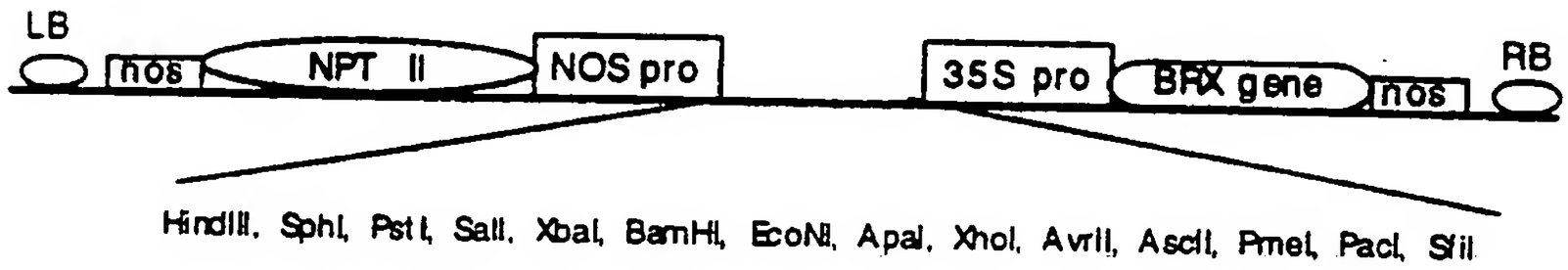
LB
nos
NPT II
NOS pro
35S pro
BRX gene
nos
RB
HindIII, SphI, PstI, SalI, XbaI, BamHI, EcoNI, ApaI, XhoI, AvrII, AscI, PmeI, PacI, SfiI

Fig. 5

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/FR 98/02375

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/435 C12N15/62 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR 2 733 237 A (RHONE POULENC AGROCHIMIE) 25 October 1996 cited in the application page 4, ligne 20-21; page 8, ligne 16 - page 9, ligne 11; revendications ---	1-30
A	FEHLBAUM, P., ET AL.: "structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 93, February 1996, pages 1221-1225, XP002071913 see the whole document ---	1-30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 1999	25/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/FR 98/02375

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	FR 2 732 345 A (RHONE POULENC AGROCHIMIE) 4 October 1996 see page 5, line 15 - line 21	1-30
A	EP 0 798 381 A (NAT INST AGROBIO RES) 1 October 1997 page 6, ligne 8-20; examples, revendications	1-30
A	WO 90 14098 A (PLANT GENETIC SYSTEMS NV) 29 November 1990 page 3, page 6, ligne 5 -18; page 6, ligne 34 - page 7, ligne9; examples, revendications	1-30
A	FEHLBAUM P ET AL: "INSECT IMMUNITY" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 52, 30 December 1994, pages 33159-33163, XP002061373 see the whole document	1-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/FR 98/02375

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2733237 A	25-10-1996	NONE	
FR 2732345 A	04-10-1996	NONE	
EP 0798381 A	01-10-1997	JP 9252779 A	30-09-1997
		JP 10028487 A	03-02-1998
		CA 2198920 A	26-09-1997
WO 9014098 A	29-11-1990	AU 5662990 A	18-12-1990

RAPPORT DE RECHERCHE INTERNATIONALE

De. Je internationale No
PCT/FR 98/02375

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/82 C07K14/435 C12N15/62 C12Q1/68 A01H5/00

Selon la classification internationale des brevets (CIB) ou a la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultee (système de classification suivi des symboles de classement)
CIB 6 C12N C07K C12Q A01H

Documentation consultee autre que la documentation minimale dans la mesure ou ces documents relèvent des domaines sur lesquels a porte la recherche

Base de donnees electronique consultee au cours de la recherche internationale (nom de la base de donnees, et si realisable, termes de recherche utilises)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Categorie *	Identification des documents cités, avec, le cas echeant, l'indication des passages pertinents	no. des revendications visees
A	FR 2 733 237 A (RHONE POULENC AGROCHIMIE) 25 octobre 1996 cité dans la demande page 4, ligne 20-21; page 8, ligne 16 - page 9, ligne 11; revendications ---	1-30
A	FEHLBAUM, P., ET AL.: "structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 93, février 1996, pages 1221-1225, XP002071913 voir le document en entier ---	1-30

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[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiques en annexe

* Categories speciales de documents cites:

"A" document définissant l'état general de la technique, non considere comme particulièrement pertinent

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"X" document particulièrement pertinent: l'invention revendiquée ne peut être consideree comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolement

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"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a ete effectivement achevee	Date d'expedition du present rapport de recherche internationale
18 février 1999	25/02/1999

Nom et adresse postale de l'administration chargee de la recherche internationale	Fonctionnaire autorise
Office Europeen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

Formulaire PCT/ISA/210 (deuxieme feuille) (juillet 1992)

RAPPORT DE RECHERCHE INTERNATIONALE

De e internationale No
PCT/FR 98/02375

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	FR 2 732 345 A (RHONE POULENC AGROCHIMIE) 4 octobre 1996 voir page 5, ligne 15 - ligne 21 ---	1-30
A	EP 0 798 381 A (NAT INST AGROBIO RES) 1 octobre 1997 page 6, ligne 8-20; examples, revendications ---	1-30
A	WO 90 14098 A (PLANT GENETIC SYSTEMS NV) 29 novembre 1990 page 3, page 6, ligne 5 -18; page 6, ligne 34 - page 7, ligne9; examples, revendications ---	1-30
A	FEHLBAUM P ET AL: "INSECT IMMUNITY" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 52, 30 décembre 1994, pages 33159-33163, XP002061373 voir le document en entier -----	1-30

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Demande internationale No
PCT/FR 98/02375

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
FR 2733237 A	25-10-1996	AUCUN	
FR 2732345 A	04-10-1996	AUCUN	
EP 0798381 A	01-10-1997	JP 9252779 A	30-09-1997
		JP 10028487 A	03-02-1998
		CA 2198920 A	26-09-1997
WO 9014098 A	29-11-1990	AU 5662990 A	18-12-1990